Animal Models of Painful Diabetic Neuropathy: The STZ Rat Model

Diabetes mellitus is one of the most common and serious chronic diseases in the United States. About 16 million or 6% of Americans have diabetes, with 5.4 million of those unaware that they have it. The prevalence of the disease has increased steadily in the last half of the twentieth century, with ~800,000 new diagnoses each year.

Neuropathy is one of the most common complications of diabetes. An estimated 50% of diabetics develop some form of peripheral neuropathy, with ~32% suffering from chronic, severe, and unremitting pain. The most frequently described manifestation of diabetic neuropathy is a distal symmetrical polyneuropathy. Patients with diabetic neuropathy can exhibit a variety of aberrant sensations, including spontaneous pain (pain in the absence of an overt stimulus) and one or more kinds of stimulus-evoked pain, including allodynia (exaggerated pain sensations to normally nonpainful stimuli) and hyperalgesia (exaggerated pain sensations to normally painful stimuli). These symptoms are often concurrent with a paradoxical loss of stimulus-evoked sensation.

The streptozotocin (STZ)-induced diabetic rat is the most commonly employed animal model used to study mechanisms of painful diabetic neuropathy and to evaluate potential therapies. The specific STZ protocol described here is used in many laboratories and is the one recommended by the Michigan Diabetes Research and Training Center at the University of Michigan. In this method, diabetes is experimentally induced in adult rats by administering a single low dose of the cytotoxic agent STZ. The resulting diabetes is similar to type I insulin-dependent diabetes mellitus (IDDM) in humans: animals injected with STZ become hyperglycemic within 72 hr, and begin to exhibit behavioral signs consistent with the development of a painful neuropathy by four weeks from the onset of diabetes.

As with neuropathic pain models involving traumatic injury to a peripheral nerve (*UNIT 9.14*), rats with STZ-induced diabetic neuropathy develop abnormalities of pain sensation that are readily assessed by quantitative behavioral testing. The behavioral assays most commonly employed in the STZ diabetic neuropathy model test for mechanical allodynia (see Support Protocol 2) and heat hyperalgesia (see Support Protocol 3). Although diabetic humans often report the presence of ongoing spontaneous pain, at present there are no reliable behavioral assays of spontaneous pain in this animal model.

NOTE: All protocols using live animals must first be reviewed and approved by an Institutional Animal Care and Use Committee (IACUC). Because of significant ethical implications involved with the induction of a chronic neuropathic pain state, in addition to the regulations of the National Institutes of Health and applicable federal laws, such research should adhere to the guidelines for pain research in animals adopted by the International Association for the Study of Pain (Zimmermann, 1992).

INDUCTION OF DIABETES WITH STZ

BASIC PROTOCOL

STZ is injected into food-deprived rats and the animals are then monitored for blood glucose levels (see Support Protocol 1).

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Current Protocols in Neuroscience (2004) 9.18.1-9.18.11

Materials

Adult rats (e.g., ~225-g Sprague-Dawley; see Critical Parameters)
STZ (see recipe)
Sodium citrate buffer, pH 5.5 (see recipe)
10% sucrose (Sigma) in tap water (store up to 3 days at 5° to 10°C)

Animal scale
Conical test tube or equivalent with screw top, sterile
Aluminum foil
1-ml TB or insulin syringes with 26- to 28-G needles
Cages with solid floors and soft bedding

Additional materials and reagents for measuring blood glucose levels (see Support Protocol 1)

1. Deprive adult rats of food (i.e., fast) 4 to 6 hr prior to STZ induction.

   This will facilitate i.p. absorption of the drug. See Critical Parameters for information concerning diet and the development of neuropathic pain symptoms.

2. Weigh all animals using an animal scale.

3. Just prior to use (i.e., within 15 to 20 min), prepare STZ solution as follows:

   a. Pour contents of one premeasured microcentrifuge tube of STZ into a sterile conical test tube covered with aluminum foil and containing sufficient sodium citrate buffer, pH 5.5, to yield a 50 mg/ml solution.
   b. Mix until STZ is completely dissolved.
   c. Store protected from light for no more than 15 to 20 min.

   CAUTION: *STZ is cytotoxic to both humans and animals. Preparation of this solution should be performed in a class II biosafety cabinet/hood that has been certified for preparation of cytotoxic materials. Prep and injection areas should be covered with disposable absorbent covers. Individuals handling these materials should follow standard precautions and wear a laboratory coat, mask, and gloves.*

   NOTE: STZ degrades rapidly in sodium citrate buffer and is sensitive to light.

4. Draw the 50 mg/ml STZ solution into a 1-ml insulin or TB syringe with 26- to 28-G needle so that the final dosage is 45 mg/kg rat. Immediately inject i.p. Repeat for each animal in the experimental group.

   Inject the rat immediately after filling the syringe to minimize exposure of the syringe contents to light.

5. House injected rats in cages with solid floors and soft bedding.

   See Critical Parameters for animal housing.

6. Supply rats with 10% sucrose water as the sole water source for 48 hr after STZ injection.

   Sucrose water protects the rats from the sudden hypoglycemic period that occurs immediately after the lysis of the pancreatic islet cells by STZ.

7. Measure body weights and blood glucose levels at 72 hr after STZ injection and subsequently at 1-week intervals to identify the onset and continued presence of diabetic hyperglycemia (see Support Protocol 1).

   Normal blood glucose levels in the rat range from 60 to 100 mg/dl. Diabetic hyperglycemia is defined as a nonfasting plasma glucose concentration >250 mg/dl. Blood glucose levels in STZ-injected animals will typically range from 300 to 550 mg/dl.

8. Assess painful neuropathy by testing for mechanical allodynia (see Support Protocol 2) and heat hyperalgesia (see Support Protocol 3), starting at ~4 weeks after induction of diabetes.

MEASUREMENT OF BLOOD GLUCOSE

SUPPORT PROTOCOL 1

Because the primary indicator for the successful induction of type I diabetes using this animal model is the development of hyperglycemia, it is necessary to measure the level of glucose in the blood circulation. Diabetic hyperglycemia is defined as a nonfasting plasma glucose concentration >250 mg/dl. Past experience with this model suggests that elevated blood glucose levels remain relatively stable and typically range from 300 to 550 mg/dl in STZ-injected animals (see Fig. 9.18.1A). Blood glucose levels in a normal control rat range from 60 to 100 mg/dl. In this model of diabetes, blood glucose should initially be measured 72 hr after STZ injection to ensure that a hyperglycemic state has developed. If blood glucose is not >250 mg/dl by 72 hr, the rat should be excluded from the diabetic group. While the frequency of subsequent blood glucose measurements is at the discretion of the investigator, an experimental protocol should always include testing for hyperglycemia at 4 weeks post-STZ, the time when neuropathic pain symptoms typically become statistically significant. Blood glucose measurements should also be performed on the day prior to any behavioral assessment for pain abnormalities. In this way, the investigator can be sure that the rat is still diabetic at the time of behavioral evaluation and that the level of hyperglycemia has been adequately maintained. In the author's laboratory, blood glucose is measured once each week for the duration of a study.

Materials

Rat injected with STZ or vehicle (see Basic Protocol 1)
Glucometer and test strips (e.g., LifeScan OneTouch Ultra Blood Glucose Meter)
Terry cloth hand towel or equivalent
Lancets (e.g., OneTouch Ultra microlancets)

1. Insert test strip in glucometer.
2. Restrain rat by wrapping in a soft towel, leaving ~3 to 5 cm at the tip of the tail exposed for blood collection.

   Restraining the rat in a towel and covering its head reduces struggling by the animal during blood collection, thus minimizing the stress placed on the animal.

3. Load a lancet into the lancing device supplied with the glucometer.
4. Gently press the end of the lancing device onto the surface of the skin near the tip of the exposed tail. Press the button on the lancing device to prick the tail and permit blood flow.

   A small drop of blood should appear on the skin surface.

   The lancet method of blood sampling is identical to that used by human diabetics, except that blood is sampled from the tail instead of the finger. The use of a lancet for blood collection instead of the more traditional tail snip procedure is much less stressful to the animal and provides a reliable sampling method that is quick, easy, and less messy.

5. Touch the sample end (active region) of the glucometer test strip into the drop of blood.
6. Wait for glucometer to analyze the blood sample (~5 sec) and record the plasma glucose level in milligrams per deciliter.

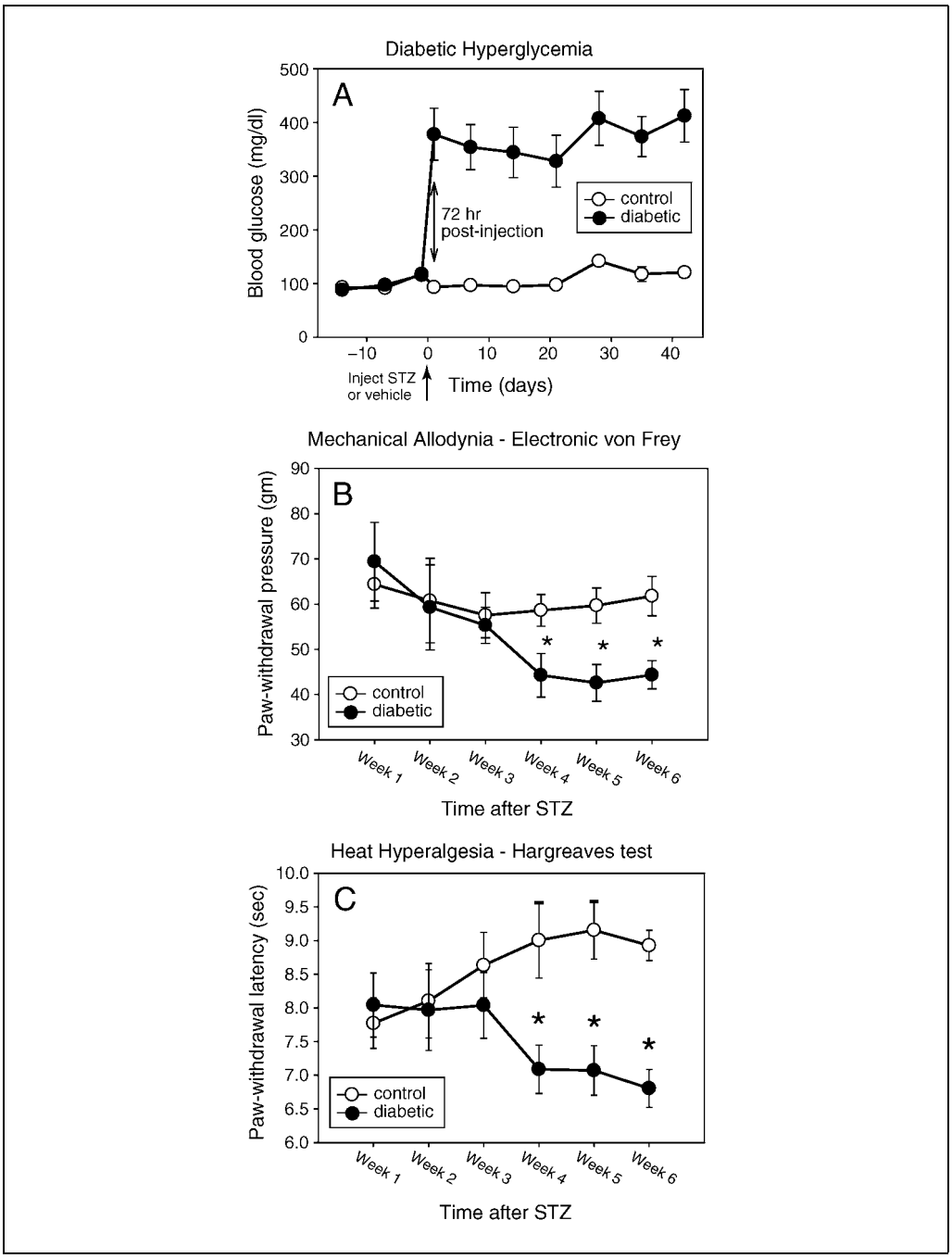

Figure 9.18.1 STZ-treated Sprague-Dawley rats develop a stable level of hyperglycemia (**A**) and neuropathic pain responses to investigator-applied mechanical and thermal stimuli. Approximately four weeks after STZ treatment, diabetic rats clearly exhibit a significant reduction in (**B**) the withdrawal threshold to mechanical pressure and (**C**) the latency to withdrawal from a noxious thermal stimulus as compared to responses of nondiabetic vehicle-injected controls. Vehicle-injected control animals showed no significant changes in behavioral response. An asterisk (∗) indicates a significant difference from control ($p \leq 0.05$ by ANOVA).

SUPPORT PROTOCOL 2

QUANTIFICATION OF MECHANICAL ALLODYNIA: THE ELECTRONIC VON FREY METHOD

Mechanical allodynia can be assessed using standard von Frey hairs or by using an electronic von Frey pressure algometer. For information on testing for mechanical allodynia with the standard Semmes-Weinstein von Frey hairs, see *UNIT 9.14*. As electronic von Frey devices have become more available, this method has increasingly become the

one preferred for testing mechanical sensitivity, because the device is not affected by the environmental conditions that can affect standard von Frey hairs.

To quantify mechanical sensitivity of the paw in pre- and post-diabetic rats, the brisk paw withdrawal in response to normally innocuous mechanical stimuli is measured as described by Möller et al. (1998). Responsiveness to mechanical stimuli is tested with a calibrated electronic von Frey pressure algometer (Somedic Sales AB). This system consists of a hand-held electronic pressure transducer fitted with a stainless steel von Frey probe with a circular tip. Tip diameters of 0.25 to 2.0 mm are available; for testing neuropathic pain in rats, the author uses a 0.5-mm-diameter probe. The algometer is connected to a computer for data collection, allowing an online display of the absolute applied force as well as the rate of stimulus application.

For testing, the rat is placed in a hanging cage with a metal mesh floor (with holes ~1-cm diameter) and allowed to acclimate for 10 min. The von Frey probe is manually applied to the plantar surface of the hind paw so that pressure increases at a rate of ~0.05 N/sec, a stimulation procedure that is easily learned by an experimenter with a little practice. The computer converts the applied stimulus intensity into grams, and the pressure at which a paw withdrawal occurs is recorded. On a given test day and for each hind paw, the same procedure is repeated five times. Successive stimuli are applied at ~30-sec intervals, alternating feet between stimuli. Because the first withdrawal on each side during baseline (pre-diabetes) and post-diabetes testing can differ markedly from subsequent responses in the session, the mean withdrawal pressure for each paw is computed by averaging the last four out of the five measurements. A significant decrease in the pressure needed to elicit a brisk paw withdrawal in response to this mechanical stimulus is interpreted as mechanical allodynia.

Electronic von Frey devices are now available from several sources. Somedic Sales AB and IITC Life Sciences both offer devices with a hand-held probe that is used to manually apply the probe tip with increasing pressure. More recently, Ugo-Basile has introduced an automated device that uses a computer-controlled motor-driven von Frey filament (probe) to apply pressure at a user-selectable rate. Unlike hand-held probe devices, the Ugo-Basile device eliminates the need to train users to manually apply the probe stimulus at a consistent repeatable rate, and thus reduces potential investigator-induced variability in the animal's behavioral responses. All of these electronic von Frey devices automatically log the pressure at which the animal's paw is withdrawn from the stimulus.

ASSESSMENT OF HEAT HYPERALGESIA

SUPPORT PROTOCOL 3

Hargreaves Apparatus (Paw-Flick Test)

Heat hyperalgesia can be assessed with the paw-flick (Hargreaves) test (also see *UNIT 9.14*) using an apparatus like that described by Hargreaves et al. (1988). This type of device is commercially available from a number of suppliers, including IITC Life Science Instruments, Ugo-Basile, Stoelting, and the Department of Anesthesiology at the University of California, San Diego (*http://anes-som.ucsd.edu*). Although they are similar in basic operation, they differ significantly in overall appearance, especially with respect to the stimulus source and photocell detector. In addition, because some versions of the Hargreaves apparatus do not employ a mechanism to maintain the temperature of the glass floor (see later discussion), it is recommended that the system used for testing always be reported.

To quantify thermal sensitivity, rats are placed in a clear Plexiglas chamber (10 × 20 × 10 cm) located on an elevated floor of clear glass (2 mm thick) and given 5 to 10 min to acclimate. A radiant heat source (i.e., 50 W, 8 V, CXL/CXP halogen projector lamp),

contained in a movable holder beneath the glass floor, delivers a heat stimulus to the plantar surface of the hind paw. When at rest, the rat will be "back on its heels," i.e., with its weight shifted backwards and the whole plantar surface in contact with the floor. Because it is critical that only the skin in good contact with the glass be stimulated, one should apply the stimulus to the fat part of the heel that lies distal to the medial and lateral pads, avoiding the long calcaneus. A reflective photocell sensor detects when the rat moves/lifts its paw, automatically shutting off the radiant heat and logging the time between onset of the stimulus and paw flick (withdrawal). This time is defined as the paw withdrawal latency.

The stimulus intensity of the light source is adjustable using a variable potentiometer and is typically set so a normal subject withdraws its stimulated hind foot at ~10 to 12 sec. If the paw is not withdrawn within 20 sec, the stimulus is automatically terminated to avoid tissue damage. Successive stimuli are alternately applied to the right or left paw until each paw is tested five times with 3 min between stimulations of either paw to avoid peripheral sensitization effects. Because the first latency from each side during baseline (pre-diabetes) and post-diabetes test sessions is typically longer than all subsequent latencies in the session, the mean withdrawal latency for each paw is computed by averaging the last four of the five measurements. A significant decrease in the paw withdrawal latency as compared to baseline latency is interpreted as heat hyperalgesia.

It is crucial that the temperature of the glass floor of the chamber be maintained at $30^\circ \pm 1^\circ$C, which is close to the normal temperature of a rat's hind paw (measured by radiometer) in a thermally neutral environment. If the glass is colder, it will markedly cool the paw and inflate the withdrawal latency. The rest of the chamber is maintained at room temperature.

Fixed-Temperature Contact Heat Stimulator

Behavioral testing with contact heat provides another method of detecting heat hyperalgesia when the use of the Hargreaves-type apparatus is unavailable or is not technically feasible because of other experimental constraints (e.g., during brain imaging studies). Contact thermal stimulators are commercially available from several sources, including Cygnus, Medoc Advanced Medical Systems, and Somedic Sales AB.

For testing with a fixed-temperature contact heat probe, the rat is restrained in a soft cotton terry cloth towel, providing gentle restraint while permitting access to both hind feet. While the restrained rat is supported by the investigator's hand, the plantar surface of one hind paw is placed against a feedback-controlled fixed-temperature (49°C) heat probe, and the latency to paw withdrawal is measured. (While there may be some experimenter-induced error introduced by this method, data from the author's laboratory show only a very small standard deviation and do not indicate a problem.) Stimuli are applied for no longer than 15 sec to avoid potential tissue damage. As with the paw-flick test, differences in resting paw temperature can influence withdrawal latencies; therefore, ambient room temperature should be the same during all test sessions and maintained in a neutral range, minimizing extremes. Successive stimuli are alternately applied to the right or left paw until each paw is tested five times with 3 min between stimulations of either paw to avoid peripheral sensitization effects. As in the paw-flick test, the mean withdrawal latency for each paw is computed by averaging the last four of the five measurements. A significant decrease in paw withdrawal latency compared to baseline latency is interpreted as heat hyperalgesia.

REAGENTS AND SOLUTIONS

Use deionized, distilled water in all recipes and protocol steps. For common stock solutions, see ***APPENDIX 2A****; for suppliers, see* ***SUPPLIERS APPENDIX****.*

Sodium citrate buffer, pH 5.5

Dissolve 210.14 mg citric acid monohydrate ($C_6H_8O_7 \cdot H_2O$; mol. wt. 210.14; Sigma) in 100 ml distilled water to make a 10 mM citric acid solution. Dissolve 294.10 mg trisodium citrate dihydrate ($C_8H_5O_7Na_3 \cdot 2H_2O$; mol. wt. 294.12; Sigma) in 100 ml distilled water to make a 10 mM sodium citrate solution. Add citric acid solution to the sodium citrate solution until the pH is 5.5 as monitored by a pH meter. Prepare fresh before experiment.

STZ

Calculate the amount of streptozotocin (STZ; Sigma) needed for all injections in a single experiment, using a final dosage of 45 mg/kg/rat (based on weight measured 1 day prior to the experiment). Weigh out this amount of STZ into a microcentrifuge tube (do not add buffer). Cover the tube with aluminum foil (STZ is light sensitive) and store at −20°C in a desiccator until ready for use in the sodium citrate buffer (see recipe). The stock bottle of STZ powder should also be stored at −20°C in a desiccator.

CAUTION: *Streptozotocin is cytotoxic to both humans and animals. The weighing of STZ powder and preparation of the STZ solution should be performed in a class II biosafety cabinet/hood that has been certified for preparation of cytotoxic materials. Prep and injection areas should be covered with disposable absorbent covers. Individuals handling these materials should follow standard precautions and wear a laboratory coat, mask, and gloves.*

COMMENTARY

Background Information

Damage to peripheral somatic nerves (neuropathy) by injury, infection, disease, or as the result of surgery can often lead to bizarre and intractable abnormalities in pain perception in humans. Degeneration of the peripheral nervous system is one of the most common of the secondary complications associated with diabetes. Diabetic neuropathy encompasses a diverse range of nerve disorders that may be broadly divided into focal and symmetrical neuropathies. Focal neuropathies are usually a consequence of compression or ischemic lesions to a specific nerve, whereas symmetrical neuropathy suggests a widespread metabolic or physiologic etiology. The most frequently described manifestation of diabetic neuropathy is a distal symmetrical polyneuropathy. Patients with diabetic neuropathy may exhibit a variety of aberrant somatic sensations, including spontaneous pain and one or more kinds of stimulus-evoked pain, including allodynia (exaggerated pain sensations to normally nonpainful stimuli) and hyperalgesia (exaggerated pain sensations to normally painful stimuli), that are often concurrent with a paradoxical loss of stimulus-evoked sensation (Benbow et al., 1994). These disorders in pain sensation occur in both type I (insulin-dependent) and type II (non-insulin-dependent) diabetics, suggesting that the disorders are not related to insulin deficiency per se (Ahroni et al., 1994). Because the pathophysiology of diabetic neuropathy and its painful variant remain poorly understood, the use of appropriate animal models is key to increasing understanding of nervous system complications of this disease.

Streptozotocin (STZ)-induced diabetes in the rat has become one of the most widely employed animal models used to study mechanisms in painful diabetic neuropathy and to evaluate potential therapies. Streptozotocin is a toxin extracted from *Streptomyces acromogenes* and is diabetogenic due to a selective cytotoxic action upon pancreatic beta islet cells (Rakieten et al., 1963). The STZ rat model exhibits early functional and biochemical abnormalities similar to those in human diabetic neuropathy (Sharma and Thomas, 1987). In

addition to hyperglycemia, statistically significant abnormalities in nerve conduction velocities, calcium signaling, mitochondrial function, and activation of the apoptosis cascade after four weeks of diabetes have been reported in this model (Srinivasan et al., 2000). Although morphological changes are mild in the peripheral nerves of the STZ rat, detailed morphometric examinations of peripheral nerves have shown reproducible changes such as reduction in fiber size along the proximal-distal gradient (Yagihashi, 1997).

Mechanical hyperalgesia, with a 30% to 40% reduction in nociceptive threshold, is well documented in STZ diabetic rats (Wuarin-Bierman et al., 1987; Ahlgren and Levine 1993; Courteix et al., 1993, 1994; Fox et al., 1999); however, there are conflicting reports regarding the development of heat hyperalgesia in the STZ model. While several groups describe STZ diabetic rats with heat hyperalgesia (Forman et al., 1986; Lee and McCarty, 1990, 1992; Courteix et al., 1993), others have reported thermal hypoalgesia (Fox et al., 1999; Calcutt, 2002) or unchanged behavioral responses to thermal stimulation (Khan et al., 2002). More recently, tactile allodynia has been demonstrated in STZ-diabetic animals (Calcutt et al., 1996; Calcutt and Chaplan 1997), and may be argued as being more representative of the cutaneous hyperaesthesia reported by patients with diabetes. It is unclear why some laboratories report thermal hyperalgesia in the STZ rat model and others do not. One possible explanation is differences in the specific methodological details of the behavioral test employed. Indeed, it is known that the location where a radiant or contact heat stimulus is applied to the plantar surface of the paw can lead to vastly different results. It is also well documented that animal models of pain are often confounded by species and strain differences in the behavioral assessment of pain.

It has been argued that STZ-treated rats show altered thresholds and withdrawal latencies in pain tests because they are ill and not because of the development of a painful peripheral neuropathy (Fox et al., 1999). Fox suggests that the discrepancy between the presence of mechanical allodynia and lack of thermal hyperalgesia supports the contention of ill health. However, several factors suggest that ill health is not a factor for the behavioral responses seen with the low-dose STZ protocol presented here. The decrease in withdrawal pressure or latency in response to mechanical or thermal stimuli shows that rats with diabetes, in fact, move sooner than control rats. Although diabetic rats treated with the low dose of STZ develop polyuria (increased urination) and exhibit low weight gain compared to controls, there is no significant weight loss and they otherwise appear healthy and well-groomed. Finally, under this protocol, diabetic rats developed both mechanical and thermal hyperalgesia, symptoms also described in other animal models of painful peripheral neuropathy (*UNIT 9.14*).

Alternative animal models to experimentally STZ-induced diabetes are available and include the diabetes prone BB/W rat (a well-accepted genetic model for type I diabetes) and several obese rat models used to study type II diabetes (Sima and Shafrir, 2000; Sima et al., 2000). The published literature for pain research using other models, however, is significantly smaller than for the STZ rat.

Critical Parameters

Animals

Adult male rats are preferred for the STZ model. Unless specifically required for a study, female rats should be avoided in order to minimize the potentially confounding effects of the estrus cycle on the behavioral assays. There are also data to suggest that relatively young adults (>200 g) develop more severe neuropathic pain syndromes (Chung et al., 1995). The effect of rat strain, vendor, and even the vendor's breeding colony may also have a significant influence on the development of diabetic neuropathy. There is evidence to suggest that there is an important genetic component for the reaction to nerve injuries (e.g., Shir et al., 1991, 2001; Kupers et al., 1992; Yoon et al., 1999). Significant differences between Sprague-Dawley rats from different breeding colonies have also been reported (Graham et al., 1997; Yoon et al., 1999).

Diet

The animal's diet should be well-defined and constant throughout an experiment. There is evidence that dietary factors can have a powerful influence on the development of neuropathic pain symptoms in traumatic nerve injury models (Shir et al., 1998). If possible, diets containing primarily plant protein (e.g., soy, alfalfa) should be avoided. Such diets will contain phytoestrogens, hormone-like chemicals that may influence the appearance of neuropathic pain.

Housing

Because diabetic rats develop symptoms of mechanical allodynia, they should be housed in cages with solid floors and soft bedding (e.g., sawdust as opposed to corn cobs or coarse wood chips). In addition, because diabetic rats drink excessively and develop polyuria (frequent urination), no more than two rats should be housed per cage and the cage bedding should be changed daily. Diurnal rhythms can have marked effects on behavior, so some investigators house their rats on a reversed light-dark cycle, although others do not. In either case, the animals should always be tested at the same time of day to minimize these effects. The temperature of the room should be carefully controlled. The behavioral tests for heat hyperalgesia are especially sensitive to changes in ambient temperature. In addition, it is possible that the sympathetic nervous system interacts with the neuropathic pain syndrome. A room that is too cold or too hot will alter the rat's basal (i.e., thermoregulatory) sympathetic discharge.

Handling and habituation

Frightened or highly stressed animals make poor subjects for quantitative behavioral testing. Before any behavioral testing is performed, new animals must be given one week to acclimate to the colony room. All animals must be thoroughly habituated to handling, to being transported between the vivarium and the laboratory, and to the testing apparatus and procedures. Animals should be kept and handled in a quiet, softly lit room. There should be minimal extraneous noise and traffic in and out of the room, since the hearing of a rat extends well into the ultrasonic range. In addition, rats should be acclimated to placement in the soft towel restraint that is used during blood glucose measurement, and to equipment used to test heat hyperalgesia.

Controls

Unlike the animal models presented in *UNIT 9.14*, where a unilateral traumatic injury to a peripheral nerve induces a mononeuropathic condition (one-sided neuropathy), diabetic rats typically develop a symmetrical polyneuropathy affecting all extremities. This means that for STZ-induced diabetic rats, there is no normal control or unaffected limb or side. While most laboratories traditionally assess the pain responsiveness of experimental animals prior to inducing a neuropathic pain state, it is common practice in some laboratories using traumatic nerve injury models to compare the behavioral responses of the injured versus uninjured sides in the same animal, rather than the pre- and post-injury responses. Since it must be assumed that sensory responses in all limbs are abnormal in the STZ diabetic neuropathy model, it is necessary to perform prediabetic (pre-STZ) behavioral testing. Comparison with this data is the only way to determine if a rat has developed symptoms of neuropathic pain due to diabetes. If one compares data obtained from diabetic versus nondiabetic rats, as in Figure 9.18.1, it is necessary to inject all control animals with vehicle, to account for any differences in behavioral response that might be induced by either the vehicle or the injection procedure alone.

Troubleshooting

The most common problem encountered in the STZ model is the failure to induce diabetic hyperglycemia. It is possible that not all injected rats will become hyperglycemic, since not all injected animals will develop diabetes from the low-dose STZ protocol described here (see Anticipated Results).

The failure to induce diabetes in all or most of the STZ-injected rats indicates there is a problem in technique. This usually occurs because the stock STZ powder was stored improperly or because the STZ powder, STZ solution, or STZ-filled syringe(s) were exposed to room light for an extended period of time. If problems inducing diabetic hyperglycemia are encountered, check that the stock STZ powder was stored in a desiccator at −20°C, protected from light, according to the manufacturer's recommendations. If not, replace the STZ, since it may have degraded. If the STZ powder was stored properly, prepare fresh citrate buffer and STZ/citrate solution for injection. Ensure that the new solution is shielded from exposure to room light. If STZ will be drawn up into multiple syringes prior to injecting rats (not recommended), place the filled syringes in a light-tight container until the injections are performed. Keep careful track of the time when the STZ/citrate solution was prepared; remember, STZ degrades after ∼15 to 20 min in solution.

Anticipated Results

With the protocol described in this unit, Sprague-Dawley rats that develop STZ-induced diabetes exhibit a relatively stable level of hyperglycemia that does not require insulin supplement for survival. Peripheral neuropathy and ultimately a chronic painful

condition will become evident ~4 weeks after induction of diabetic hyperglycemia (Fig. 9.18.1). As mentioned above, mechanical hyperalgesia and allodynia are common features of STZ-induced painful diabetic neuropathy, whereas there are conflicting reports regarding the development of heat hyperalgesia. It is unclear why some investigators report heat hyperalgesia and others do not. The presence or failure to develop or identify abnormal responses to noxious heat stimuli may depend on the specific details of how the behavioral assay is performed, or on the specific strain, substrain, or breeding source of animal used in the study.

The low dose of STZ used in this protocol will induce diabetes in only ~70% to 80% of the injected Sprague-Dawley rats. Higher-dose STZ protocols are reported in the literature, but they do produce diabetes in a higher percentage of injected animals. Furthermore, these animals have been described as "sickly" (Fox et al., 1999), exhibiting significant weight loss and showing signs of both liver and kidney toxicity. These animals are therefore less appropriate for studying neuropathic pain in diabetic neuropathy.

Time Considerations

Preparation of the sodium citrate buffer solution used to dissolve the STZ requires ~20 to 30 min. Preparation of the final STZ/sodium citrate buffer solution requires another 10 to 15 min. Injection of four to six rats with STZ solution requires ~8 to 10 min. Glucose measurements are done at 3 days following injection and at 1-week intervals thereafter, and require 1 to 2 min per rat. Behavioral assessment is typically begun 3 to 4 weeks after inducing diabetes. For a group of four to six rats, a thermal hyperalgesia assay (Hargreaves test or contact heat) can be done in ~45 min and a mechanical allodynia assay (von Frey Test) can be done in ~30 min.

Literature Cited

Ahlgren, S.C. and Levine, J.D. 1993. Mechanical hyperalgesia in streptozotocin-diabetic rats. *Neuroscience* 52:1049-1055.

Ahroni, P., Boyko, E.J., Davignon, D.R., and Pecson, R.D. 1994. The health and functional status of veterans with diabetes. *Diabet. Care* 17:319-321.

Benbow, J., Chan, A.W., Bowsher, D., MacFarlane, I.A., and Williams, G. 1994. A prospective study of painful symptoms, small-fibre function and peripheral vascular disease in chronic painful diabetic neuropathy. *Diabet. Med.* 17-21.

Calcutt, N.A. 2002. Potential mechanisms of neuropathic pain in diabetes. *Int. Rev. Neurobiol.* 50:205-228.

Calcutt, N.A. and Chaplan, S.R. 1997. Spinal pharmacology of tactile allodynia in diabetic rats. *Br. J. Pharmacol.* 122:1478-1482.

Calcutt, N.A., Jorge, M.C., Yaksh, T.L., and Chaplan, S.R. 1996. Tactile allodynia and formalin hyperalgesia in streptozotocin- diabetic rats: Effects of insulin, aldose reductase inhibition and lidocaine. *Pain* 68:293-299.

Chung, J.M., Choi, Y., Yoon, Y.W., and Na, H.S. 1995. Effects of age on behavioral signs of neuropathic pain in an experimental rat model. *Neurosci. Lett.* 183:54-57.

Courteix, C., Eschalier, A., and Lavarenne, J. 1993. Streptozocin-induced diabetic rats: Behavioural evidence for a model of chronic pain. *Pain* 53:81-88.

Courteix, C., Bardin, M., Chantelauze, C., Lavarenne, J., and Eschalier, A. 1994. Study of the sensitivity of the diabetes-induced pain model in rats to a range of analgesics. *Pain* 57:153-160.

Forman, L.J., Estilow, S., Lewis, M., and Vasilenko, P. 1986. Streptozocin diabetes alters immunoreactive beta-endorphin levels and pain perception after 8 wk in female rats. *Diabetes* 35:1309-1313.

Fox, A., Eastwood, C., Gentry, C., Manning, D., and Urban, L. 1999. Critical evaluation of the streptozotocin model of painful diabetic neuropathy in the rat. *Pain* 81:307-316.

Graham, B.A., Hammond, D.L., and Proudfit, H.K. 1997. Differences in the antinociceptive effects of alpha-2 adrenoceptor agonists in two substrains of Sprague-Dawley rats. *J. Pharm. Exp. Therapeut.* 283:511-519.

Hargreaves, K., Dubner, R., Brown, F., Flores, C., and Joris, J. 1988. A new sensitive method for measuring thermal nociception in cutaneous hyperalgesia (PAI 01152). *Pain* 32:77-88.

Khan, G.M., Chen, S.R., and Pan, H.L. 2002. Role of primary afferent nerves in allodynia caused by diabetic neuropathy in rats. *Neuroscience* 114:291-299.

Kupers, R.C., Nuytten, D., De Castro-Costa, M., and Gybels, J.M. 1992. A time course analysis of the changes in spontaneous and evoked behaviour in a rat model of neuropathic pain. *Pain* 50:101-111.

Lee, J.H. and McCarty, R. 1990. Glycemic control of pain threshold in diabetic and control rats. *Physiol. Behav.* 47:225-230.

Lee, J.H. and McCarty, R. 1992. Pain threshold in diabetic rats: Effects of good versus poor diabetic control. *Pain* 50:231-236.

Möller, K.A., Johansson, B., and Berge, O.G. 1998. Assessing mechanical allodynia in the rat paw with a new electronic algometer. *J. Neurosci. Methods* 84:41-47.

Rakieten, N., Rakieten, L., and Nadkarni, M.V. 1963. Studies on the diabetogenic action of

streptozotocin. *Cancer Chemother. Rep.* 29:91-98.

Sharma, A.K. and Thomas, P.K. 1987. Animal models: Pathology and pathophysiology. *In* Diabetic Neuropathy (P.J. Dyck, P.K. Thomas, A.K. Asbury, A.I. Winegard, and D. Porte Jr., eds.) pp. 237-252. Saunders, Philadelphia.

Shir, Y., Raber, P., Devor, M., and Seltzer, Z. 1991. Mechano- and thermo-sensitivity in rats genetically prone to developing neuropathic pain. *Neuroreport* 2:313-316.

Shir, Y., Zeltser, R., Vatine, J.J., Carmi, G., Belfer, I., Zangen, A., Overstreet, D., Raber, P., and Seltzer, Z. 2001. Correlation of intact sensibility and neuropathic pain-related behaviors in eight inbred and outbred rat strains and selection lines. *Pain* 90:75-82.

Sima, A.A. and Shafrir, E. (eds.) 2000. Animal Models in Diabetes: A Primer, pp. 320. Taylor & Francis Group, London.

Sima, A.A.F., Zhang, W., Xu, G., Sugimoto, K., Guberski, D., and Yorek, M.A. 2000. A comparison of diabetic polyneuropathy in Type II diabetic BBZDR/Wor rats and in Type I diabetic BB/Wor rats. *Diabetologia* 43:786-793.

Srinivasan, S., Stevens, M., and Wiley, J.W. 2000. Diabetic peripheral neuropathy—Evidence for apoptosis and associated mitochondrial dysfunction. *Diabetes* 49:1932-1938.

Wuarin-Bierman, L., Zahnd, G.R., Kaufmann, F., Burcklen, L., and Adler, J. 1987. Hyperalgesia in spontaneous and experimental animal models of diabetic neuropathy. *Diabetologia* 30:653-658.

Yagihashi, S. 1997. Nerve structural defects in diabetic neuropathy: Do animals exhibit similar changes? *Neurosci. Res. Commun.* 21:25-32.

Yoon, Y.W., Lee, D.H., Lee, B.H., Chung, K., and Chung, J.M. 1999. Different strains and substrains of rats show different levels of neuropathic pain behaviors. *Exp. Brain Res.* 129:167-171.

Zimmermann, M. 1992. Ethical constraints in pain research. *In* Animal Pain: Ethical and Scientific Perspectives (T.R. Kuchel, M. Rose, and J. Burrell, eds.) pp. 13-18. Australian Council on the Care of Animals in Research and Teaching, Glen Osmond, SA, Australia.

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